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Serum microRNA181a: Correlates with the intracellular cytokine levels and a potential biomarker for acute graft-versus-host disease

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ABSTRACT

The aim of this study was to investigate the clinical relevance of lymphocyte-related serum miRNAs to the pathogenesis of acute graft-versus-host disease (aGVHD) and evaluate the predictive and prognosis value of miRNAs. Consecutive patients who received allogeneic peripheral blood stem cell transplantation (allo-PBSCT) in General Hospital of Jinan Military District were enrolled. aGVHD patients were diagnosed and graded clinically, and divided into the training set and the testing set. Blood samples were collected, total RNA was isolated, and RT-PCR was performed for miRNA expression (miR-181a-3p, miR-214-3p and miR-326). Intracellular cytokines levels were assayed by flow cytometry, and the disease specificity assay of miRNAs for aGVHD was detected. A total of 120 patients were admitted. Serum level of miR-181a in aGVHD patients was highly increased and associated with the severity of aGVHD, but not miR-214 and miR-326. Levels of cytokines including IL-2, IL-22, and IL-17a were positively correlated with miR-181a level, while serum IL-13 level was negatively correlated with miR-181a level in aGVHD patients. Moreover, increased miR-181a level was not detected in patients with acute rejection after kidney transplantation or sepsis patients. MiR-181a level was sensitively and specifically increased, especially in severe aGVHD patients. MiR-181a may be a potential biomarker for the identification, diagnosis, and prognosis of aGVHD patients.

1. Introduction

Acute graft-versus-host disease (aGVHD) is a common and life-threatening complication that develops within 100 days after allogeneic peripheral blood stem cell transplantation (allo-PBSCT) [1]. It is defined histologically as degenerative, systemic and destructive changes by immuno-suppression in organs, such as skin, liver, lung, gastrointestinal tract, and bone marrow, and it increases the transplant-related morbidity and mortality rates to 90% [2]. Diagnosis of aGVHD is mainly based on clinical symptoms in main target organs and biopsy results [3]. However, few validated diagnostic or predictive blood biomarker for aGVHD has been found and used in clinical decision making [4,5], nor the combined application of multiple biomarkers. Therefore, it is necessary to explore useful biomarkers for the diagnosis and treatment of aGVHD.

MicroRNAs (miRNAs) are a class of small noncoding 21–23 nt RNAs that play crucial roles in regulating gene expression and inducing messenger RNA degradation [6]. Previous studies referred that miRNAs are present in plasma, serum, urine, and other body fluids in a remarkably stable form for various diseases such as cancer, sepsis, liver injury and infection, and organ transplant rejection, including aGVHD [7–9]. Zhou et al. reported that serum miR-155 was a potential therapy biomarker for aGVHD diagnosis [10]. Nelson and his colleagues proved that miR-423, miR-199a-3p, miR-93, and miR-377 may serve as independent biomarkers for aGVHD diagnosis, prediction, and prognosis [11]. In addition, increasing evidences suggest that miRNAs are involved in various autoimmune diseases and immune responses. Weitzel et al. proved that miR-184 can regulate the expression of NFAT-dependent cytokine IL-2 via affecting the protein expression in aGVHD [12]. In addition, serum cytokine level of IL-17a was found to be positively correlated with miR-155 expression level in aGVHD patients [10]. Despite many studies have devoted increasing attention to explore the roles of miRNAs in the immune system, role of them in aGVHD has not been fully discussed; still, more miRNAs involving in the pathogenesis of aGVHD should be discovered.

Over-expressed miR-181a could enhance activation-dependent signaling in human neonatal naive $CD4^+$ T cells which is associated

Abbreviations: GVHD, graft-versus-host disease; allo-PBSCT, allogeneic peripheral blood stem cell transplantation; OD, optical density; HD, healthy donors; ROC, receiver-operating characteristic.

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with lower incidence of aGVHD [13], while miR-326 was found regulating Th17 cell development to contribute to CD4-mediated GVDH [14]. Moreover, miR-214 was also found to be associated with Th17 cell differentiation via TGF-β and mTOR signaling in $CD4^+$ T cells [15]. Thus we suspected that these three miRNAs may also be involved in some functions in the molecular pathogenesis of aGVDH. In this pilot study, we retrospectively analyzed the serum expression patterns of three miRNAs in aGVHD and non-aGVHD patients. Cytokines including IL-17a, IL-2, IL-6, IL-10, IL-13, IL-22, TNF-α and IFN-γ as proinflammatory mediators have been found to be correlated with aGVHD [16]. Thus the associations between aGVHD-related miRNAs and cytokine were also explored. Moreover, the relationship between aGVHD and miRNAs was validated in other disease entities. This study may help to identify potential novel biomarkers for aGVHD prediction and prognosis.

2. Materials and methods

All subjects provided written informed consent in accordance with the approval of Jinan Military General Hospital and the Ethics Committee of General Hospital of Jinan Military District before participation.

2.1. Patients and groups

During January 2009 and June 2014, all consecutive patients received allogeneic PBSCT due to hematologic malignancies and non-malignancies at General Hospital of Jinan Military District were enrolled in this study. Patients who receive T-cell depleted graft or umbilical cord blood transplantation, or who had primary graft failure or previous graft rejection were excluded. aGVHD was diagnosed and graded clinically with the aid of histological confirmation according to the established criteria [17]. All available clinical and pathologic GVHD organ staging data of all patients were retrospectively reviewed. All aGVHD patients were divided into 2 groups based on maximal aGVHD grade: low grade (I–II) and high grade (III–IV). All patients with aGVHD received initial therapy and additional immunosuppressive therapy [18].

To investigate the reproducibility of the relationship between miRNA and aGVHD and to look for consistency in preliminary results, the consecutive patients received allogeneic PBSCT between January 2009 and December 2012 were chosen as the training set, while the other patients (between January 2013 and June 2014) were chosen as the blinded testing and validation set.

2.2. Blood sample collection and RNA extraction

The whole blood was drawn two days before, within 24 h after, and every two week till 6 months after allogeneic PBSCT. Moreover, blood samples were obtained weekly after the diagnosis of aGVHD. Plasma and serum samples from each patient were frozen at −20 °C. In this study, the blood samples obtained within 24 h after allogeneic PBSCT, at the time of diagnosis and at the maximal aGVHD grade were used for later analyses, while the last blood sample of non-aGVHD patients was analyzed.

Total RNAs were extracted from 400 μL of serum using the mirVana PARIS Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The concentration and optical density (OD) 260/280 ratio of RNA were quantified with a spectrophotometer (Eppendorf, Hamburg, Germany), and RNA samples were reverse transcribed with MMLV Reverse Transcriptase (Promega, USA) according to the manufacturers' instructions.

2.3. Quantitative real-time RT-PCR

To investigate whether miRNA expression is related to aGVHD in patients, miRNAs (miR-181a-3p, miR-214-3p and miR-326) levels in serum were analyzed in aGVHD patients and in non-aGVHD patients using SYBR Green-based quantitative reverse transcriptase polymerase chain reaction as previously described [19]. Essential miRNA-specific data are presented in Table 1. Each

Table 1
Information of patients suffered from acute graft-versus-host disease (GVHD).

Characteristic	Discovery and training phase			Validation and blinded tested phase		
	Non-GVHD (n = 37)	GVHD (n = 52)	p	Non-GVHD (n = 15)	GVHD (n = 16)	p
Median age (range)	27 (13–51)	25.5 (14–52)		29 (17–49)	28 (15–51)	
Disease			0.22			0.56
Malignant	31	45		11	11	
Others	6	7	0.12	4	5	0.81
Disease status at PBSCT						
Low/mediate risk	15	19		3	4	
High risk	22	33		8	7	
Donor type			0.61			0.19
Related	9	13		4	4	
Unrelated	28	39		11	12	
Regimen type			0.46			0.39
Nonmyeloablative	5	5		3	3	
Myeloablative	32	47		12	13	
Maximum GVHD grade						
I–II		33			12	
⩾III		19			4	
Organ target at GVHD onset						
Skin		10			5	
Gut		8			3	
Liver		12			5	
Multiple organs		22			3	
GVHD diagnosis day after PBSCT						
First 15 day		10			2	
Day 16 to day 42		26			9	
After day 43		16			5	

Malignant diseases include acute myelocytic leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia, myelodysplastic syndrome. Other disease include aplastic anemia, paroxysmal nocturnal hemoglobinuria (PNH) and Fanconi anemia. PBSCT: peripheral blood stem cell transplantation.